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The 5′CL-PCBP RNP complex, 3′ poly(A) tail and 2A^{pro} are required for optimal translation of poliovirus RNA

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ABSTRACT

In this study, we showed that the 5′CL-PCBP complex, 3′ poly(A) tail and viral protein 2A^{pro} are all required for optimal translation of PV RNA. The 2A^{pro}-mediated stimulation of translation was observed in the presence or absence of both the 5′CL and the 3′ poly(A) tail. Using protein–RNA tethering, we established that the 5′CL-PCBP complex is required for optimal viral RNA translation and identified the KH3 domain of PCBP2 as the functional region. We also showed that the 5′CL-PCBP complex and the 3′ poly(A) tail stimulate translation independent of each other. In addition to the independent function of each element, the 5′CL and the 3′ poly(A) tail function synergistically to stimulate and prolong translation. These results are consistent with a model in which the 5′CL-PCBP complex interacts with the 3′ poly(A)-PABP complex to form a 5′–3′ circular complex that facilitates ribosome reloading and stimulates PV RNA translation.

Introduction

Poliovirus (PV) belongs to the *Picornaviridae* family of single-stranded positive-sense RNA viruses. The PV genome contains a large open reading frame that is flanked by the 5′ NTR, which includes the internal ribosome entry site (IRES), and the 3′ NTR and poly(A) tail. A small viral protein, VPg, is covalently linked to the 5′ end of the genome (Flanegan et al., 1977; Lee et al., 1977; Ambros & Baltimore, 1978; Pettersson et al., 1978; Wimmer et al., 1993). Translation of the viral RNA genome is directed by the IRES and takes place in the cytoplasm of the infected cell (Pelletier et al., 1988; Pelletier & Sonenberg, 1988; Pelletier & Sonenberg, 1989). Translation of the viral genomic RNA results in the synthesis of a polyprotein which is cleaved by the viral proteases, 2A^{pro} and 3C^{pro}/3CD^{pro} (Krausslich & Wimmer, 1988; Harris et al., 1990).

The terminal 5′ cloverleaf (5′CL), IRES, 3′ NTR and poly(A) tail are important *cis*-active RNA elements that regulate different steps in the PV life cycle. In general, these *cis*-active elements function in the form of ribonucleoprotein complexes (RNP) which contain both viral and cellular proteins. The 5′CL is organized into stem *a* and stem-loops 'b', 'c' and 'd,' where stem-loops 'b' and 'd' bind the cellular poly(C) binding proteins (PCBP) and viral protein 3CD^{pro}, respectively (Andino et al., 1990; Andino et al., 1993; Parsley et al., 1997). Recently, a PCBP binding site was also identified in the C-rich sequence adjacent to the 5′CL (Toyoda et al., 2007). Previous studies show that mutations in stem-loop 'b' including those that specifically disrupt PCBP binding to stem-loop 'b' result in the inhibition of PV RNA translation (Simoes & Sarnow, 1991; Parsley et al., 1997; Gamarnik & Andino, 1998; Lyons et al., 2001). However, these results are complicated by the fact that disrupting PCBP binding to the 5′CL also destabilizes PV RNA (Murray et al., 2001). Other studies report that the presence of a 3′ poly(A) tail stimulates IRES-driven translation of reporter RNA constructs, and the stimulation of translation is observed in the absence of 2A^{pro} (Bergamini et al., 2000; Michel et al., 2001; Svitkin et al., 2001; Dobrikova et al., 2006).

The viral protease 2A^{pro} is a critical protein involved in many aspects of the PV life cycle. 2A^{pro} is a cysteine protease which catalyzes the primary cleavage of the polyprotein, separating the capsid protein precursor (P1) from the replication protein precursor (P23) (Toyoda et al., 1986; Hellen et al., 1989, 1992). Aside from its function in viral polyprotein processing, 2A^{pro} inhibits host cell protein synthesis through the cleavage of the eukaryotic initiation factor eIF4G, which leads to the inactivation of the cap-binding complex (eIF4F) (Etchison et al., 1982; Lamphear et al., 1995; Borman et al., 1997). Although cap-dependent translation of cellular mRNAs is inhibited, viral translation does not require intact eIF4G and therefore, is not inhibited by 2A^{pro} (Ziegler et al., 1995; Belsham & Sonenberg, 1996). In addition to the inhibition of host protein synthesis, 2A^{pro} has been shown to stimulate the translation of both entero- and rhinovirus IRES-driven translation (Hambidge & Sarnow, 1992; Ziegler et al., 1995; Borman et al., 1997; Roberts et al., 1998; Svitkin et al., 2001; Dobrikova et al., 2006). In a previous study from our laboratory, we showed that

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proteolytically active $2A^{pro}$ directly stimulates and prolongs PV RNA translation, in addition to its ability to stabilize PV RNA. Furthermore, we showed that $2A^{pro}$ and the $2A^{pro}$ containing precursor proteins, 2AB and P2, were the only PV encoded proteins that were able to stimulate and prolong PV RNA translation (Jurgens et al., 2006).

In the present study, we used HeLa S10 translation–replication reactions to define the role of the 5′CL-PCBP complex, 3′NTR, poly(A) tail and $2A^{pro}$ in the translation of PV RNA. We found that the presence of the 5′CL-PCBP complex, the 3′ poly(A) tail and $2A^{pro}$ stimulated PV RNA translation. In contrast, the 3′ NTR had no effect on translation independent of the poly(A) tail. By tethering PCBP2 to the 5′CL, we further established the importance of the 5′CL-PCBP complex in enhancing viral RNA translation. Taken together, the 5′CL-PCBP complex, 3′ poly(A) tail and $2A^{pro}$ were all required to observe optimal levels of PV RNA translation. In addition, our results support a model in which the formation of a 5′–3′ circular RNP complex facilitates ribosome reloading and enhances translation.

Results

In this study, we used a poliovirus subgenomic transcript RNA, PV1p50 RNA, to examine the requirements for optimal translation of PV RNA (Fig. 1). PV1p50 RNA (p50 RNA) contains an in-frame deletion in the coding region in PV RNA and encodes a 50-kDa nonfunctional protein (p50), which serves as a reporter protein for monitoring the translation of PV RNA. A significant advantage of using p50 RNA to characterize the translation of PV RNA is the inclusion of the authentic 5′ and 3′ NTRs and the authentic viral translation initiation and termination sequences. In addition, the labeled protein synthesized in these reactions can be easily quantitated. Protein synthesis was measured by pulse-labeling for 1-h intervals over a period of 4 h and the amount of labeled p50 synthesized during each hour was determined by gel electrophoresis and autoradiography (Fig. 2A). The amount of labeled p50 protein synthesized during each hour was quantitated and is shown in Fig. 2B. This allowed us to measure both the rate of protein synthesis during each hour of the reaction and the total amount of protein synthesized in each reaction.

Role of 5′CL in PV RNA translation

To characterize the role of the 5′CL in translation of PV RNA, we used a 5′CL mutation (C24A) which is known to inhibit the binding of PCBP to stem-loop 'b' (Andino et al., 1993; Murray et al., 2001; Lyons et al., 2001). Since the C24A mutation destabilizes PV RNA (Murray et al., 2001), we used RNA transcripts with a 5′ cap to restore the stability of this mutant RNA as described in Materials and methods. In the reaction which contained p50 RNA, p50 protein synthesis continued for the entire 4 h reaction (Fig. 2A, lanes 1–4 and B). The largest amount of p50 synthesized was observed between 1 and 2 h and gradually decreased between 2–3 h and 3–4 h (Fig. 2A, lanes 1–4 and B). In contrast, in reactions containing C24A RNA, p50 was only synthesized in significant amounts from 0–1 h and 1–2 h (Fig. 2A, lanes 5–8 and B). There was a dramatic decrease in the rate of protein synthesis after 2 h and only very small amounts of p50 were

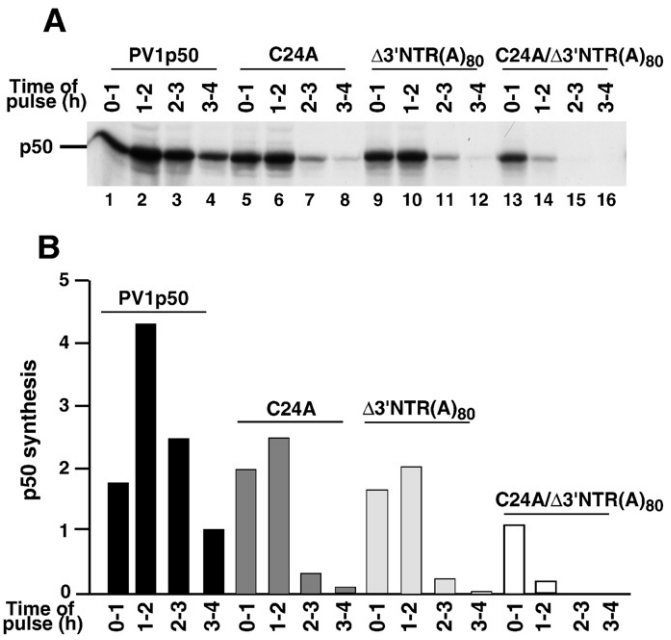

Fig. 2. Requirement of the 5′CL-PCBP complex and 3′NTR$(A)_{80}$ for efficient PV RNA translation. (A) Translation of either PV1p50, PV1p50(C24A), PV1p50(Δ3′NTR$(A)_{80}$) or PV1p50(C24A/Δ3′NTR$(A)_{80}$) RNA at a concentration of 34 μg/ml was measured by pulse-labeling for 1 h over a period of 4 h at 34 °C. The reactions were pulse-labeled with 15 μCi [^{35}S]methionine for 1 h at the indicated time points. At the end of the pulse, 4 μl of the translation reaction was solubilized in 40 μl SDS sample buffer. Labeled p50 protein synthesized was analyzed by 9–18% SDS-PAGE and visualized by autoradiography. (B) The amount of labeled p50 synthesized during each hour of the pulse was quantitated using a PhosphorImager.

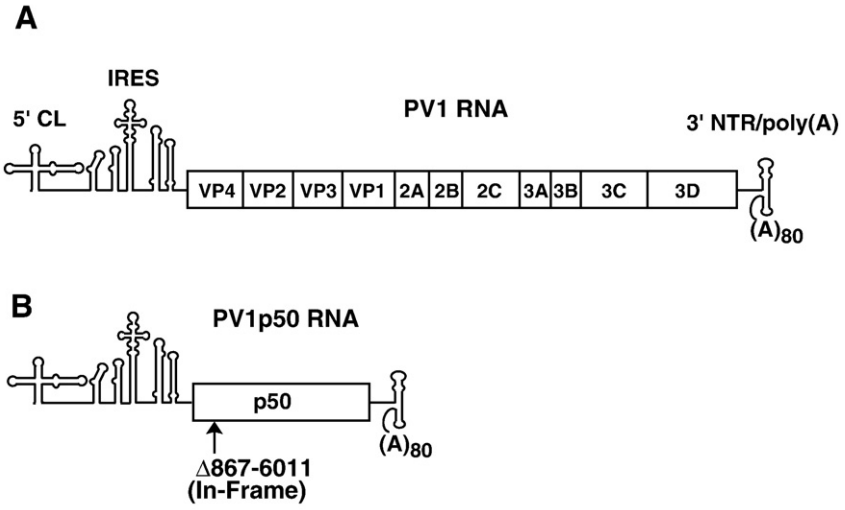

Fig. 1. Schematic of poliovirus RNAs utilized in this study. (A) Diagram of the full-length PV1 RNA which encodes all of the viral proteins. (B) Diagram of PV1p50 RNA in which nucleotides 867–6011 from PV1 RNA are deleted. This RNA contains the authentic 5′ NTR, IRES, 3′ NTR and poly(A) tail of PV1 RNA. It also contains the authentic initiation and stop codons of the viral polyprotein.

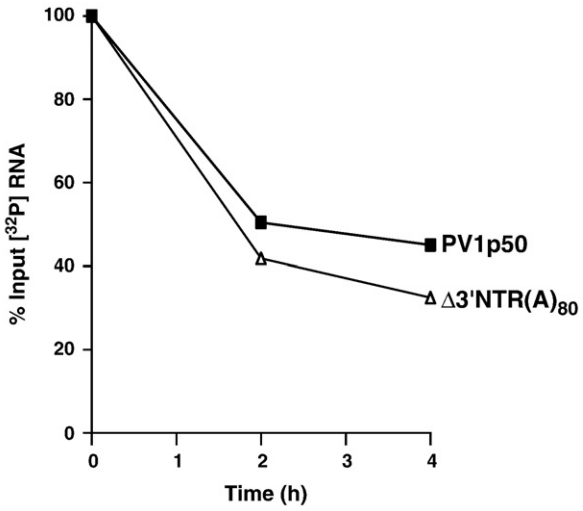

Fig. 3. RNA stability of PV1p50 and PV1p50(Δ3′NTR(A)$_{80}$) RNA in HeLa S10 reactions. ^{32}P-labeled PV1p50 or PV1p50(Δ3′NTR(A)$_{80}$) RNAs were added to reactions at a final concentration of 34 μg/ml and incubated for 4 h at 34 °C. Samples were removed at the indicated time points and the amount of labeled RNA that remained intact was determined by precipitation in tricholoroacetic acid. The amount of labeled RNA recovered at each time point was calculated as a percentage of the amount of input RNA.

synthesized from 2–3 h and 3–4 h (Fig. 2A, lanes 7–8 and B). The total amount of p50 synthesized in reactions containing C24A RNA from 0 to 4 h was about 50% of that observed with p50 RNA. The levels of translation observed in reactions that contained an RNA in which the 5′CL (nt1–88) was deleted (Δ5′CL RNA) were similar to those observed in reactions containing C24A RNA (data not shown). Taken together, these results indicated that the presence of the 5′CL, and specifically the 5′CL-PCBP complex, was required for optimal translation of PV RNA (Fig. 2).

Role of 3′NTR-poly(A) tail in PV RNA translation

To determine the role of the 3′NTR-poly(A) tail in the translation of PV RNA, the translation of p50(Δ3′NTR(A)$_{80}$) RNA was compared to the translation of p50 RNA. Since deletion of the poly(A) tail could potentially destabilize the RNA, a small hairpin was engineered at the 3′ end of the p50(Δ3′NTR(A)$_{80}$) RNA to stabilize the RNA as described in Materials and methods. In RNA stability assays, we observed that the amount of p50(Δ3′NTR(A)$_{80}$) RNA that remained intact during the course of the reaction was only about 10% less than the amount observed with wildtype RNA (Fig. 3). Therefore, the presence of the small 3′ terminal hairpin effectively stabilized the p50(Δ3′NTR(A)$_{80}$) RNA. In the reaction which contained p50 RNA, p50 protein synthesis continued for the entire 4 h reaction as described above (Fig. 2A, lanes 1–4 and B). In reactions containing Δ3′NTR(A)$_{80}$ RNA, significant amounts of p50 protein were synthesized from 0–1 h and 1–2 h. After 2 h, however, only very low levels of p50 synthesis were observed (Fig. 2A, lanes 9–12 and B). The total amount of p50 synthesis in reactions containing Δ3′NTR(A)$_{80}$ RNA from 0 to 4 h was about 40% of the amount observed with p50 RNA. These results indicated that the deleting the 3′-NTR-poly(A) tail significantly inhibited translation of PV RNA (Fig. 2).

Independent and synergistic effects of the 5′CL and 3′ poly(A) tail on PV RNA translation

To determine whether the 5′CL and 3′ poly(A) tail function as independent and/or coupled translational enhancers, we engineered p50 RNA, which contained both the C24A and the Δ3′NTR(A)$_{80}$ mutations. The translation of the p50(C24A/Δ3′NTR(A)$_{80}$) RNA was compared to the translation of p50 RNA, C24A RNA and Δ3′NTR(A)$_{80}$ RNA. In reactions containing C24A/Δ3′NTR(A)$_{80}$ RNA, protein synthesis was severely inhibited after the first hour (Fig. 2A, lanes 13-16 and B). The total amount of p50 synthesized in reactions containing C24A/Δ3′NTR(A)$_{80}$ RNA from 0 to 4 h was approximately 15% of that observed with p50 RNA. The overall level of translation observed with the double-mutant RNA was significantly lower than that observed with either C24A RNA or Δ3′NTR(A)$_{80}$ RNA (Fig. 2). The level of translation observed in reactions containing C24A/Δ3′NTR (A)$_{80}$ RNA, in essence, represents the baseline for translation in these experiments. The presence of either the 5′CL or the 3′ poly(A) tail stimulated translation above this baseline level from 0 to 2 h (Fig. 2B). These results indicated that each RNA element (i.e., the 5′CL or the 3′ poly(A) tail) can function independently of each other to stimulate translation from 0 to 2 h. In addition, it is important to note that only in the presence of both the 5′CL and 3′ poly(A) tail does translation continue at high levels beyond 2 h (Fig. 2A, lanes 3–4, 7–8 and 11–12 and B). The level of translation observed beyond 2 h with the p50 RNA does not appear to result from an additive effect of the independent stimulation observed with the 5′CL and the 3′ poly(A) tail alone. For example, the level of translation observed between 2 and 3 h in the p50 RNA reaction was four-fold higher than the sum of the translation levels observed in the reactions containing the C24A and the Δ3′NTR (A)$_{80}$ RNAs. Between 3 and 4 h, the translation in the p50 reaction was eight-fold higher than the sum of the translation in the C24A and Δ3′ NTR(A)$_{80}$ RNA reactions (Fig. 2B). Therefore, these findings indicated that in addition to their independent function, the 5′CL and 3′poly(A) tail also function synergistically to stimulate and prolong translation.

The poly(A) tail independent of the 3′NTR stimulates PV RNA translation

Previous studies have shown that translation of PV RNA is not affected by the deletion of the 3′ NTR (Brown et al., 2005). This suggested that the poly(A) tail, not the 3′ NTR, enhanced PV RNA translation. To confirm that the 3′ poly(A) tail alone was responsible

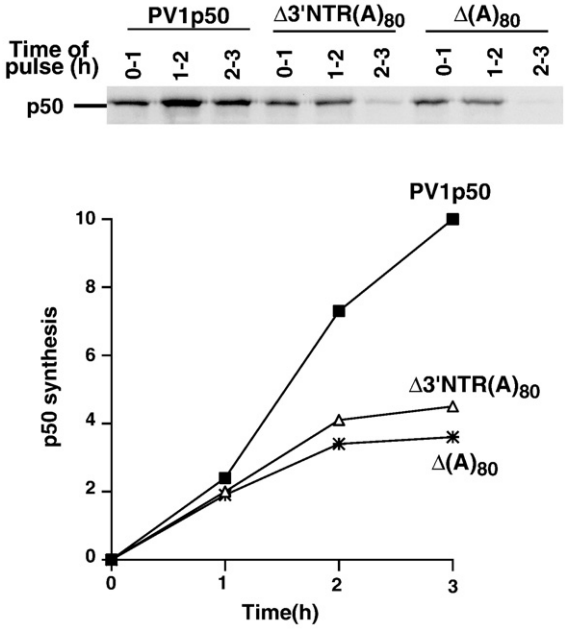

Fig. 4. Requirement of the 3′ poly (A) tail, independent of the 3′NTR, for efficient PV RNA translation. Translation of either PV1p50, PV1p50(Δ3′NTR(A)$_{80}$) or PV1p50 (Δ(A)$_{80}$) RNA at a concentration of 34 μg/ml was measured by pulse-labeling for 1 h over a period of 3 h as described in Fig. 2. The amount of labeled protein synthesized during each hour of the pulse was quantitated using a PhosphorImager. To quantify the cumulative level of protein synthesized, the amount of labeled protein synthesized during each hour of the pulse was added to the previous total during the 4-h time period and expressed as a function of time.

for enhancing translation, we measured the translation of p50 ($\Delta(A)_{80}$) RNA, in which only the poly(A) tail was deleted. The translation observed in reactions containing $\Delta(A)_{80}$ RNA was compared to that observed with $\Delta 3'NTR(A)_{80}$ RNA and p50 RNA (Fig. 4). As expected, no significant difference was observed between $\Delta(A)_{80}$ RNA and $\Delta 3'NTR(A)_{80}$ RNA in either the duration or overall levels of translation. Based on these findings, we concluded that the 3′ poly(A) alone was required to enhance the translation of PV RNA.

Tethering PCBP2 to the 5′CL stimulates PV RNA translation

To directly investigate the ability of the 5′CL-PCBP to enhance PV RNA translation, we used a protein–RNA tethering system which has been described in more detail in a separate study (Spear et al., 2008). Briefly, the PCBP binding site in stem loop 'b' of the 5′CL was replaced with the cognate binding site for the MS2 bacteriophage coat protein dimer ($(MS2)_2$). After this change, the endogenous PCBP in HeLa S10 reactions no longer binds to the modified 5′CL ($5'CL^{MS2}$). By expressing $(MS2)_2$PCBP2 fusion protein in HeLa S10 reactions, the PCBP2 fusion protein can be effectively tethered to the RNA which contains the $5'CL^{MS2}$, via the $(MS2)_2$ protein–RNA interaction (Fig. 5). Therefore, functional studies relative to the 5′CL-PCBP complex can be performed in the presence of endogenous PCBP, without affecting the PCBP-IRES interaction, which is required for translation initiation.

The translation of either p50 RNA or p50($5'CL^{MS2}$) RNA was measured in the presence of a second RNA which encoded either $(MS2)_2$ or $(MS2)_2$PCBP2. The $(MS2)_2$ and $(MS2)_2$PCBP2 proteins were expressed at similar levels and were stable in these reactions (see Materials and methods) (Spear et al., 2008). No significant difference in the cumulative level of p50 synthesis was observed in reactions containing p50 RNA and either the $(MS2)_2$ or $(MS2)_2$PCBP2 expression RNAs (Fig. 5A). As expected, in reactions containing 5′ CL^{MS2} RNA, the translation of $5'CL^{MS2}$ RNA was significantly reduced compared to that observed with p50 RNA (Fig. 5A and B). The cumulative level of protein synthesis in reactions containing $5'CL^{MS2}$ RNA and $(MS2)_2$ expression RNA was approximately 50% of the levels observed in the p50 RNA controls (compare Fig. 5A and B). However, when PCBP2 was tethered to the $5'CL^{MS2}$ RNA by expression of the $(MS2)_2$PCBP2 fusion protein, a significant increase in protein synthesis was observed compared to the level observed in the reaction containing the $(MS2)_2$ protein (Fig. 5B). The cumulative level of protein synthesized in the presence of the $(MS2)_2$PCBP2 protein was approximately 80% of the level observed with the p50 RNA controls (compare Fig. 5A and B). These results clearly demonstrated that PCBP2, when tethered to the 5′CL, stimulated PV RNA translation.

The KH3 region of PCBP2 stimulates PV RNA translation

Given the efficacy of the MS2 protein–RNA tethering system in establishing the requirement for the 5′CL-PCBP2 complex in the efficient translation of viral RNA, we used this assay to identify the critical region of PCBP2 that is required for optimal translation. The PCBP family is characterized by the presence of three conserved hnRNP-K homology domains (KH Domains) (Gibson et al., 1993; Siomi et al., 1993). To identify the functional domain of PCBP2, the coding region of PCBP2 was divided into three regions, each containing one of

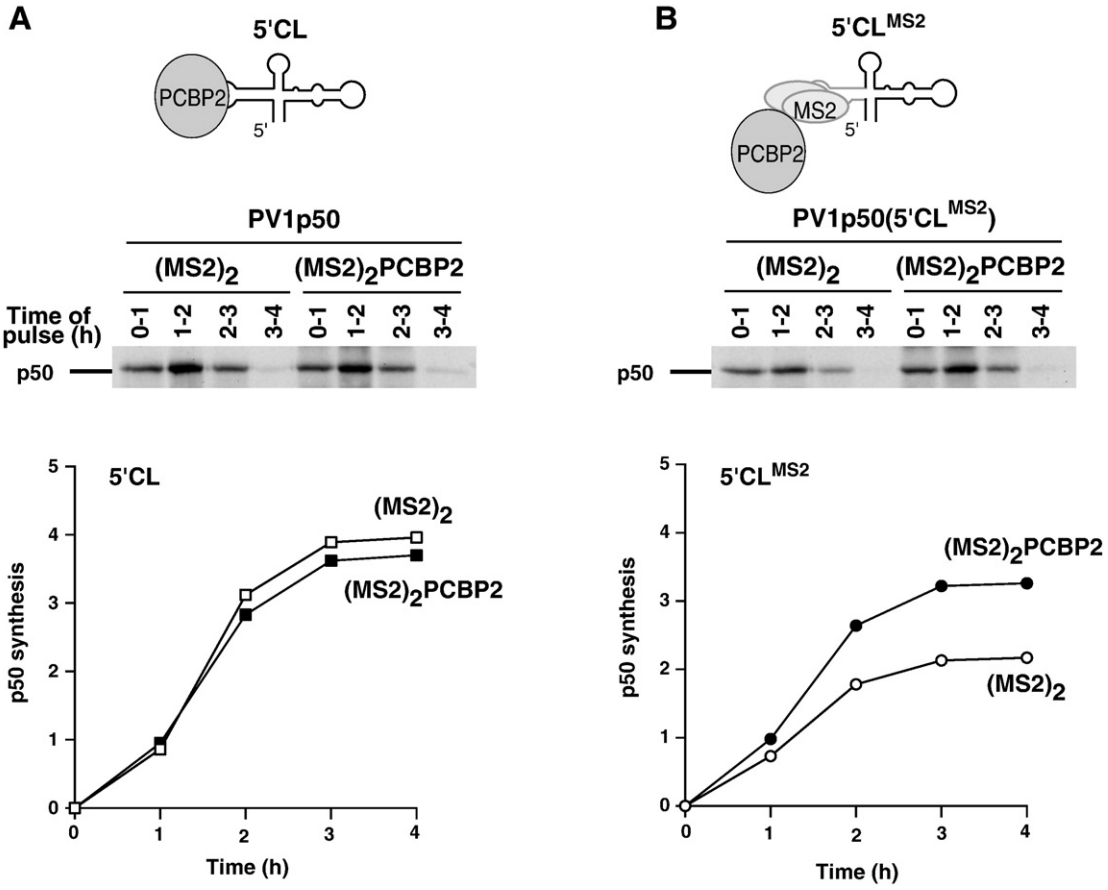

Fig. 5. Tethering PCBP2 to the 5′CL enhances PV RNA translation. Translation of (A) PV1p50 RNA and (B) PV1p50($5'CL^{MS2}$) RNA was measured in the presence of either $(MS2)_2$ or $(MS2)_2$PCBP2 fusion protein expression RNA. Stem-loop 'b' in PV1p50($5'CL^{MS2}$) RNA was replaced with the cognate binding site of the MS2 protein and is bound by $(MS2)_2$ or $(MS2)_2$PCBP2 fusion protein. The amount of labeled protein synthesized during the 4-h time period was calculated and depicted as described for Fig. 4. An equimolar RNA ratio was maintained in these reactions. The reactions shown in panels (A) and (B) were performed at the same time and analyzed on the same gel.

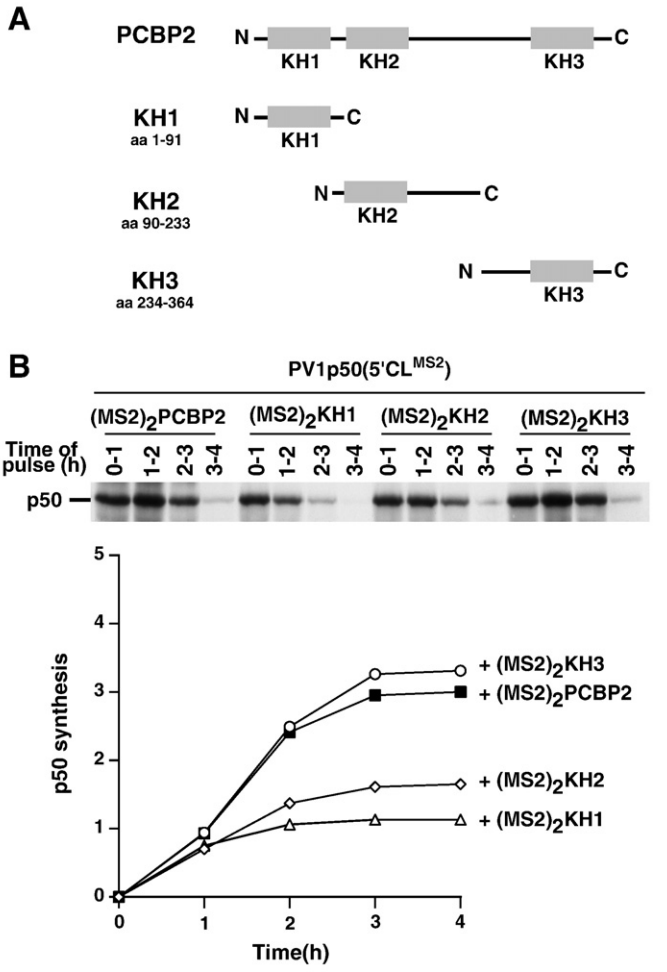

Fig. 6. Tethering the KH3 region of PCBP2 to the 5′CL enhances PV RNA translation (A) Schematic of the structure of full length PCBP2 containing the three KH domains and the individual protein fragments containing each of the KH domains. (B) Translation of PV1p50(5′CL^{MS2}) RNA was measured in the presence of either $(MS2)_2$PCBP2, $(MS2)_2$KH1, $(MS2)_2$KH2 or $(MS2)_2$KH3 fusion protein expression RNA as before. The amount of labeled protein synthesized during the 4-h time period was calculated and depicted as described for Fig. 4. An equimolar RNA ratio was maintained in these reactions.

the three KH domains (Fig. 6A) (Spear et al., 2008). The coding sequence for each of these regions was fused to the $(MS2)_2$ coding sequence, and translation of 5′CL^{MS2} RNA was assayed in the presence of each of the resultant fusion protein expression RNAs. There was no significant difference in the expression or stability of the individual $(MS2)_2$ fusion proteins in these reactions (see Materials and methods) (Spear et al., 2008). In reactions containing 5′CL^{MS2} RNA and either $(MS2)_2$KH1or $(MS2)_2$KH2, the level of p50 synthesis was significantly lower than that observed in the presence of $(MS2)_2$PCBP2 (Fig. 6B). In contrast, in reactions that contained the 5′CL^{MS2} RNA and $(MS2)_2$KH3 the level of p50 synthesis was approximately equal to the level observed in the presence of $(MS2)_2$PCBP2 (Fig. 6B). These results indicated that the KH3 region of PCBP2 is the functional domain in PCBP2 that is responsible for the observed stimulation of PV RNA translation.

Role of the 5′CL-PCBP complex and 3′ poly(A) tail on PV RNA translation in the presence of 2A^{pro}

As shown above, both the 5′CL and the 3′ poly(A) tail stimulated translation of p50 RNA. Since 2A^{pro} is also known to stimulate PV RNA translation, it was important to determine the role of the 5′CL and the 3′ poly(A) tail on translation in the presence of 2A^{pro}. To address this question, the individual reporter RNAs were co-translated with expression RNAs encoding either 2A^{pro} or inactive 2A(C109A) (Jurgens et al., 2006). In reactions containing p50 RNA and 2A^{pro}, p50 protein synthesis was observed during the 4-h reaction. The largest amount of p50 synthesized was observed between 1 and 2 h and then decreased between 2–3 h and 3–4 h (Fig. 7A, lanes 1–4 and B; Fig. 8A, lanes 1–4 and B). In contrast, in reactions containing the C24A RNA and 2A^{pro}, significant amounts of p50 were only synthesized between 0–1 h and 1–2 h. Low levels of p50 were synthesized between 2–3 h and 3–4 h (Fig. 7A, lanes 9–12 and Fig. 7B). The overall level of translation observed in reactions containing the C24A RNA was about 55% of the level observed with p50 RNA. Likewise, in reactions containing the Δ3′NTR$(A)_{80}$ RNA and 2A^{pro}, p50 was only synthesized in significant amounts between 0–1 h and 1–2 h and low levels of p50 synthesis was observed after 2 h (Fig. 8A, lanes 9–12 and B). The overall translation level observed with the Δ3′NTR $(A)_{80}$ RNA was about 40% of the level observed with p50 RNA. Therefore, both the 5′CL-PCBP complex and the 3′ poly(A) tail played an important role in maintaining optimal levels of PV RNA translation in the presence 2A^{pro}.

We have previously shown that 2A^{pro} stimulates the translation of PV RNA independent of its effects on viral RNA stability and replication (Jurgens et al., 2006). Consistent with our previous studies, the translation of p50 RNA was significantly higher in reactions containing 2A^{pro} than in reactions containing 2A(C109A) (Fig. 7A, lanes 1–8 and Fig. 8A, lanes 1–8). Similarly, the translation of either C24A RNA or Δ3′ NTR$(A)_{80}$ RNA was significantly higher in reactions containing 2A^{pro} than in reactions containing 2A(C109A) (Fig. 7A, lanes 9–16 and Fig. 8A, lanes 9–16). The cumulative level of p50 synthesis observed in the presence of 2A^{pro}, in all cases, was about 2-fold higher than the level observed in the presence of 2A(C109A). Taken together, these results demonstrated that the 2A^{pro}-mediated stimulation of PV RNA translation did not require either the 5′CL-PCBP complex or the 3′ poly (A) tail. Therefore, 2A^{pro} enhanced translation in a manner independent of the 5′CL-PCBP complex and 3′ poly(A) tail.

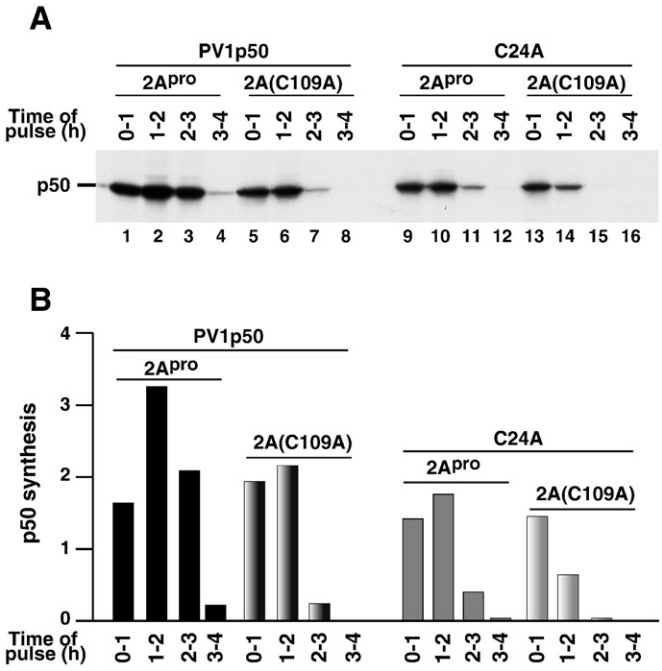

Fig. 7. Requirement of the 5′CL-PCBP complex for efficient PV RNA translation in the presence or absence of active 2A^{pro}. (A) Translation of PV1p50 and PV1p50(C24A) RNA was measured in the presence of either 2A^{pro} or 2A(C109A) RNA. The molar ratio of reporter RNA to the 2A expression RNAs was 1:2. (B) The amount of labeled p50 synthesized during each hour of the pulse was quantitated using a PhosphorImager.

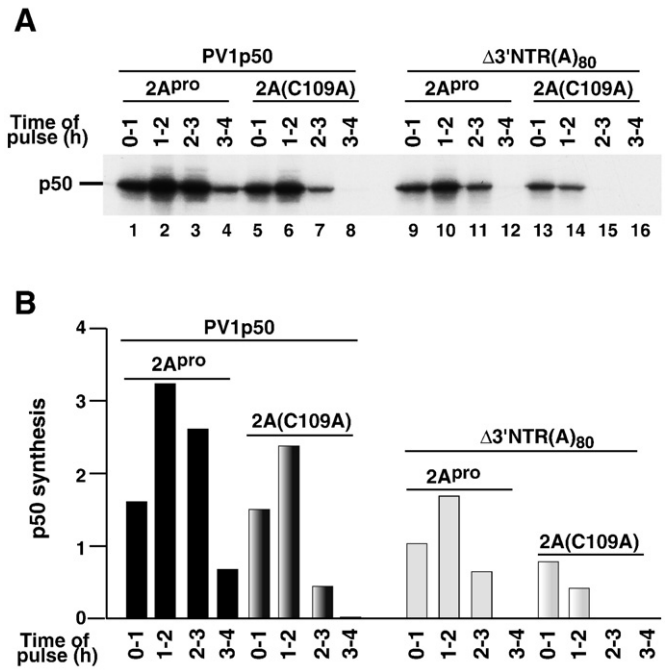

Fig. 8. Requirement of the 3′ poly(A) tail for efficient PV RNA translation in the presence or absence of active $2A^{pro}$. (A) Translation of PV1p50 and PV1p50(Δ3′NTR$(A)_{80}$) RNA was measured in the presence of either $2A^{pro}$ or 2A(C109A) RNA. The molar ratio of reporter RNA to the 2A expression RNAs was 1:2. (B) The amount of labeled p50 synthesized during each hour of the pulse was quantitated using a PhosphorImager.

Discussion

In this study, we investigated the role of the 5′CL-PCBP complex, the 3′ poly(A) tail and $2A^{pro}$ in regulating the translation of PV RNA. We translated a subgenomic PV RNA and monitored the synthesis of a reporter protein, p50. P50 synthesis was measured by pulse-labeling over a period of 4 h, which allowed us to determine both the rate and total amount of protein synthesized in each reaction. Using this approach, we showed that optimal translation of PV RNA requires the simultaneous presence of the 5′CL, 3′ poly(A) tail and $2A^{pro}$. Although the 5′CL-PCBP complex and 3′ poly(A) tail individually stimulated translation, the presence of both elements synergistically enhanced PV RNA translation. Based on this observation, we propose a model in which the formation of the 5′–3′ circular complex facilitates ribosome reloading and enhances translation.

$2A^{pro}$ stimulates translation independent of the 5′CL-PCBP complex and 3′ poly(A) tail

Previous studies from our laboratory showed that $2A^{pro}$ stimulated translation independent of its ability to stabilize PV RNA (Jurgens et al., 2006). Other studies have also shown that $2A^{pro}$ stimulates translation of PV RNA (Hambidge & Sarnow, 1992; Ziegler et al., 1995; Borman et al., 1995, 1997; Roberts et al., 1998; Kempf & Barton, 2008a). It has been proposed that the C-terminal $2A^{pro}$ cleavage fragment of eIF4G directly stimulates IRES-driven translation (Ohlmann et al., 1996; Borman et al., 1997; Svitkin et al., 2001). The results of another study, however, have shown that the expression of the two individual $2A^{pro}$ cleavage fragments of eIF4G did not enhance the translation of Coxsackievirus B3 IRES-driven translation in transfected cells (Dobrikova et al., 2006). Furthermore, active $2A^{pro}$ was recently shown to be required for both polysome maturation and to maintain the stability of polysomes during PV RNA translation (Kempf & Barton, 2008a). In the current study, we showed $2A^{pro}$ enhanced PV RNA translation in the presence or absence of the 5′CL-PCBP complex. A similar $2A^{pro}$-mediated stimulation of translation was observed in the presence or absence of the 3′ poly(A) tail. Taken together, these results suggested that $2A^{pro}$ stimulated translation initiation on the PV IRES. Although PV RNA translation is observed in the absence of $2A^{pro}$, the results of our study and others show that proteolytically active $2A^{pro}$ is required to stimulate translation and polysome formation (Hambidge & Sarnow, 1992; Ziegler et al., 1995; Borman et al., 1997; Roberts et al., 1998; Svitkin et al., 2001; Dobrikova et al., 2006; Kempf & Barton, 2008a). Our results showed that the $2A^{pro}$-mediated increase in PV RNA translation was not observed during the first hour of the reaction. This observation can be explained since some time is required for $2A^{pro}$ to be synthesized and then cleave a target cellular protein(s). Consistent with our results are recent findings from the Barton laboratory, which showed that in HeLa translation extracts complete cleavage of eIF4G1 and eIF4G11 by $2A^{pro}$ required 60 and 120 min, respectively (Kempf & Barton, 2008a). Therefore, our results and the time required to cleave eIF4G and eIF4G11 are consistent with the idea that an eIF4G cleavage product may either directly or indirectly stimulate IRES-driven translation initiation.

The 5′CL-PCBP complex stimulates PV RNA translation

PCBP is a multi-functional cellular RNA binding protein that functions in controlling the stability and translational of a number of cellular mRNAs (reviewed by Makeyev & Liebhaber, 2002). The three KH domains identified in PCBP mediate many of the RNA–protein interactions and protein–protein interactions involved in the formation of RNP complexes. Several studies have shown that PCBP2 binds to stem loop IV of the PV IRES and is an essential cofactor for poliovirus translation (Blyn et al., 1996; Gamarnik & Andino, 1997; Blyn et al., 1997; Silvera et al., 1999; Walter et al., 1999; Gamarnik & Andino, 2000; Walter et al., 2002). Previous studies have shown that all three KH domains and the linker region between KH2 and KH3 in PCBP2 play an important role in binding to the IRES and subsequent translation of PV RNA (Walter et al., 2002; Bedard et al., 2004, 2007; Sean et al., 2008). The cellular protein SRp20 was recently identified as a PCBP2 interacting protein that binds to the KH3 domain, and this complex was shown to be required for translation initiation (Bedard et al., 2007). PCBP also binds to the 5′CL and mutations that disrupt this binding partially inhibited the translation of PV transcript RNAs (Parsley et al., 1997; Gamarnik & Andino, 1998). Furthermore, recent work showed that polysome formation was decreased on PV RNA containing a mutation which disrupts PCBP binding to the 5′CL (Kempf & Barton, 2008b). However, in these studies it was difficult to discern whether the primary effect of the mutation was on RNA stability or translation, since disrupting PCBP binding to the 5′CL has been shown to destabilize viral RNA (Murray et al., 2001).

In this study, using stable transcript RNAs, we measured the effect of disrupting the 5′CL-PCBP complex on the kinetics of PV RNA translation. We showed that disrupting this complex resulted in a significant inhibition of translation compared to wildtype p50 RNA. After the first 2 h of the reaction, there was a dramatic inhibition of translation. The overall level of protein synthesis observed at 4 h was about 50% of the level observed in the presence of the 5′CL-PCBP complex. Based on these findings, we concluded that the presence of the 5′CL-PCBP complex was required for high levels of translation. Furthermore, we showed that the 5′CL-PCBP complex stimulated PV RNA translation both in the presence or absence of $2A^{pro}$. To more directly investigate the role of the 5′CL-PCBP complex in RNA translation, we utilized the $(MS2)_2$ protein–RNA tethering system to tether PCBP to the 5′CL. This system allowed us to specifically tether PCBP2 to the 5′CL without affecting the ability of PCBP to bind stem-loop IV of the IRES. Using this approach, we showed that the KH3 region of PCBP2, when tethered to the RNA, functioned as well or better than full-length PCBP2 to enhance PV RNA translation. Since KH3 does not contain any multimerization domains, multimerization of PCBP is

apparently not required for this function. These findings also demonstrated that direct binding of PCBP to the 5′CL was not required to mediate these effects. Taken together, these findings indicated that the 5′CL-PCBP complex, via the KH3 domain in PCBP2, facilitated translation initiation and thereby enhanced PV RNA translation.

3′ poly(A) tail stimulates PV RNA translation

Several studies reported that the 3′ poly(A) tail stimulates the IRES-dependent translation of reporter RNA constructs (Bergamini et al., 2000; Michel et al., 2001; Svitkin et al., 2001; Dobrikova et al., 2006; Bradrick et al., 2007). In this study, we showed that deleting the 3′ poly(A) tail resulted in a significant inhibition of translation compared to the wildtype p50 RNA. The overall level of translation observed was about 40% of the level observed in the presence of wildtype p50 RNA. Based on these findings, we concluded that the 3′ poly(A) tail was also required for high levels of translation. It has been proposed that the stimulating effect of the 3′ poly(A) tail on translation, in the absence of $2A^{pro}$, is due to the formation of a 5′–3′ circular complex between the IRES and the 3′ poly(A) tail mediated by eIF4G and PABP (Michel et al., 2001; Svitkin et al., 2001). However, in the presence of $2A^{pro}$, eIF4G would be cleaved and this 5′–3′ circular RNP complex would be disrupted. In this study, we showed that the 3′ poly(A) tail stimulated translation both in the presence and absence of $2A^{pro}$. No significant difference between the level of 3′ poly(A) tail-mediated stimulation of translation was observed in the presence or absence of active $2A^{pro}$. Therefore, these findings suggest an alternative mechanism for the stimulation of IRES-driven translation by the 3′ poly(A) tail that is not dependent on intact eIF4G.

The 5′CL and 3′NTR-poly(A) act both independently and synergistically to stimulate and prolong translation

Our findings showed that the 5′CL-PCBP complex and the 3′ poly (A) tail were both required for optimal translation. Based on these findings, we asked whether these two RNA elements function as independent and/or synergistic enhancers of viral translation. We determined the baseline level of translation in the absence of the 5′CL-PCBP complex and the 3′ poly(A) tail. Interestingly, the presence of either the 5′CL-PCBP complex or the 3′ poly(A) tail alone enhanced translation above baseline levels during the first 2 h of the reaction. This result demonstrated that the 5′CL-PCBP complex and 3′ poly(A) tail functioned independently to stimulate translation early in the reaction. At late translation times, however, it is important to note that the 5′CL-PCBP complex and the 3′ poly(A) tail were both required to observe high translation levels. Adding the independent stimulatory effect of the 5′ and 3′ RNA elements was not sufficient to explain the high levels of translation observed with wildtype (p50) RNA. Therefore, these findings indicated that the 5′CL-PCBP complex and the 3′ poly(A) tail function synergistically to stimulate and prolong translation at late times. These results are consistent with a model in which the 5′CL-PCBP complex interacts with the 3′ poly(A)-PABP complex to circularize the RNA genome to facilitate ribosome reloading at late translation times. During the first 1–2 h of the reaction, ribosomes load on to the IRES to initiate translation on the input RNA, followed by polysome formation and maturation. This is consistent with recent studies from the Barton laboratory which showed that polysome formation and maturation continued to increase during the first 60 min of translation in HeLa S10 translation reactions (Kempf & Barton, 2008a). During this early phase when polyribosomes are being formed, it is unlikely that ribosome reloading would play a significant role in stimulating translation. However, at late translation times, after mature polysomes have formed and there is limited availability of free ribosomes, ribosome reloading facilitated by 5′–3′ circularization may play an important role in prolonging translation. In contrast, to the 5′–3′ circular RNP complex used by cellular mRNAs, poliovirus may use an alternative 5′–3′ circular RNP complex to facilitate optimal levels of protein synthesis. This serves as another example of a virus evolving a novel molecular mechanism to enhance its own replication while simultaneously disrupting essential cellular functions.

Materials and methods

cDNA clones and transcript RNAs

A cDNA clone of the Mahoney strain of type I poliovirus, as pT7-PV1$(A)_{80}$ is referred to as pPV1. The RNA obtained form transcription of this clone was designated PV1 RNA (Fig. 1A). pPV1 was used as the parental clone to construct the subgenomic construct pPV1p50 used in this study. pPV1p50 (pPV1$(A)_{80}\Delta_{C867\text{-}T6011}$) contained a 5144 nucleotide (nt) deletion (Fig. 1B). The RNA obtained from transcription of this plasmid was designated PV1p50 RNA. Translation of PV1p50 RNA in HeLa S10 reactions resulted in the synthesis of a protein designated as p50 containing 494 amino acids. The p50 protein contained the amino-terminus of the viral structural protein, VP4, fused to part of C-terminus of $3D^{pol}$. pPV1p50 was used as the parental clone to construct the following plasmid constructs. (i) To construct pPV1p50Δ3′NTR$(A)_{80}$, we engineered a small hairpin (eight GC base-pairs and a GAAA tetraloop) and a Sma1 restriction enzyme site after the stop codon of $3D^{pol}$, which corresponds to nucleotide A_{7376} of pPV1$(A)_{80}$. We added this small 3′ stabilizing hairpin to stabilize the RNA because the removal of the poly(A) tail makes the RNA susceptible to degradation. (ii) To construct pPV1p50Δ$(A)_{80}$, we engineered a small hairpin (eight GC base-pairs and a GAAA tetraloop) and a NotI restriction site after nucleotide 7445 of pPV1. (iii) Plasmid pPV1p50-5′CL^{MS2} was engineered by substituting nucleotides C_{12} to G_{32} in stem-loop b of the 5′CL with the stem-loop sequence from MS2 bacterial phage RNA (ACATGAGGATTACCCATGT) that binds dimers of the MS2 capsid protein ($(MS2)_2$). RNA transcript obtained from this plasmid was designated as PV1p50-5′CL^{MS2} RNA. (iv) Plasmid pPV1p50Δ5′CL was constructed by deleting nt T_1 to A_{88} from pPV1p50. The RNA obtained from this plasmid was designated PV1p50Δ5′CL RNA. Plasmid $p2A^{pro}$ and p2A(C109A) have been previously described (Jurgens et al., 2006).

Protein expression RNAs $2A^{pro}$ and 2A(C109A) have been previously described (Jurgens et al., 2006). Translation of the $2A^{pro}$ RNA resulted in the synthesis of the $2A^{pro}$ and translation of 2A(C109A) RNA resulted in the synthesis of inactive $2A^{pro}$, where the cysteine residue in the active site of $2A^{pro}$ was mutated to alanine. Protein expression RNAs transcribed from p$(MS2)_2$, p$(MS2)_2$PCBP2, p$(MS2)_2$KH1, p$(MS2)_2$KH2 and p$(MS2)_2$KH3 were used to translate the capsid protein covalent dimer $(MS2)_2$, the fusion proteins $(MS2)_2$PCBP2, $(MS2)_2$KH1, $(MS2)_2$KH2 and $(MS2)_2$KH3 proteins respectively. The construction of these plasmids is described in a separate study (Spear et al., 2008).

RNA transcript preparation

Plasmid DNAs containing PV1 sequences were linearized with MluI except pPV1p50Δ3′NTRΔ$(A)_{80}$ and pPV1p50Δ$(A)_{80}$ which were linearized with SmaI and NotI, respectively. The linearized plasmids were transcribed in vitro in reactions containing bacteriophage T7 RNA polymerase and 500 μM of each nucleoside triphosphate (NTP) as previously described (Barton et al., 1996). We and others have shown that the presence of a 5′ cap restores the stability of PV transcript RNAs which contain a 5′CL mutation (Barton et al., 2001; Sharma et al., 2005). In particular, the presence of a 5′ cap stabilizes transcript RNAs with a C24A mutation in stem-loop 'b' of the 5′CL (Murray et al., 2001; Kempf & Barton, 2008a). Therefore, in this study we used capped transcript RNAs to control for RNA stability. In these reactions, 7-methyl guanosine cap analog (1 mM) was added to the

transcription reaction mixture and the concentration of GTP was lowered to 0.2 mM (Barton et al., 2001; Spear et al., 2008). In previous studies, it was shown that the addition of a 5′ cap had no effect on the translation of wildtype PV1 RNA (Barton et al., 2001). In all cases, the RNA transcripts were purified by Sephadex G-50 gel filtration chromatography as described previously and stored in ethanol at −20 °C (Barton et al., 1996).

RNA stability assay

Since the removal of the poly(A) tail in PV1p50Δ3′NTRΔ$(A)_{80}$ and PV1p50Δ$(A)_{80}$ transcript RNAs could potentially destabilize the RNA, a small hairpin was engineered at the 3′ end to stabilize the RNA. To confirm that the 3′ hairpin stabilized these mutant RNAs, we performed RNA stability experiments. Labeled RNAs were transcribed in reactions containing 50 μCi of [α-^{32}P]CTP (400 Ci/mmol) and were added to the HeLa S10 reactions at a concentration of 34 μg/ml. The reaction mixture was incubated at 34 °C for 4 h. At the indicated time 20 μl of the reaction mixture was removed and added to 400 μl of 0.5% SDS buffer [10 mM Tris–HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA. 0.5% sodium dodecyl sulfate]. A 50-μl portion of this reaction mixture was removed in duplicate, and labeled RNA was precipitated in 1 ml of 5% trichloroacetic acid–2% sodium pyrophosphate–5 μl of yeast tRNA (50 μg/ml). The labeled RNA was collected on filters and quantitated by liquid scintillation counting. The amount of labeled RNA recovered at each time point was calculated as a percentage of the amount of input RNA.

RNA translation assays

Ten microliters aliquots of HeLa S10 translation–replication reactions containing capped reporter RNAs were incubated at 34 °C (Jurgens et al., 2006). At the beginning of each indicated pulse, 15 μCi of [^{35}S]methionine (>1000 Ci/mmol; Amersham) was added to a 10-μl aliquot of the reaction and incubated for 1 h at 34 °C. At the end of the pulse, 4 μl of the translation reaction was solubilized in 40 μl of SDS sample buffer (Barton et al., 1996). The reaction was heated for 3 min at 100 °C and [^{35}S]methionine-labeled protein was analyzed by electrophoresis on a 9–18% SDS-polyacrylamide gel (SDS-PAGE). The gel was fixed in 40% methanol and 10% acetic acid, fluorographed using Amplify (Amersham) and dried. Labeled proteins were analyzed by autoradiography and quantitated using a PhosphorImager (Molecular Dynamics). In order to express the total protein synthesized as a function of time, the amount of labeled protein synthesized during each hour of the pulse was added to the previous total during the 4-h time period.

Where indicated, the individual expression RNAs encoding the $(MS2)_2$ protein, $(MS2)_2$PCBP2 fusion proteins, the 2A^{pro} or the 2A (C109A) proteins were co-translated with the p50 reporter RNA to synthesize the respective proteins. In the reactions containing the $(MS2)_2$ and $(MS2)_2$PCBP2 expression RNAs, equimolar amounts of the p50 reporter RNA and the expression RNA at a total RNA concentration of 50 μg/ml was added. In the reactions that contained 2A^{pro} or 2A(C109A) expression RNAs, the molar ratio of the p50 reporter RNA to the expression RNA was 1:2 and the total RNA concentration was maintained at 50 μg/ml. In these reactions, we confirmed that the $(MS2)_2$PCBP2 fusion proteins as well as the 2A^{pro} and the 2A(C109A) were stable and expressed in similar amounts in each reaction. The expression and stability of these proteins in co-translation reactions were shown in our previous studies (Jurgens et al., 2006; Spear et al., 2008). Finally, it should be noted that in the co-translation experiments, the overall level and duration of p50 synthesis is partially reduced in the presence of the second RNA. Therefore, the kinetics of p50 synthesis in reactions containing the p50 reporter RNA alone cannot be directly compared to the kinetics of p50 synthesis in the co-translation reactions.

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